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14. ABSTRACT
Breast cancer develops from breast mucosa and breast mucosa has intact immune system to maintain epithelial integrity. In this study our goal was to study the immune subsets associated with breast mucosa and develop the strategies to populate mucosa with immune effectors in order to prevent breast cancer. Data obtained from our studies suggest that T cells constitute the majority of immune cells in breast mucosa and this includes conventional CD4 T cells, CD8+ αβ T cells and significant fraction of unconventional double positive (DP) CD4+CD8+ αβ T cells. We also observed that intramammary immunization induces antigen-specific immune responses in breast mucosa. Currently, studies are being done to characterize these double positive T cells to determine whether these are regulatory or cytotoxic in nature and their roles in prevention of breast cancer. In addition to this, we are also investigating the ability of intramammary immunization in prevention of breast cancer and the feasibility of translating this approach into preventive breast cancer vaccine setting.

15. SUBJECT TERMS
Intraepithelial lymphocytes, Theilers murine encephalomyelitis virus, rat neu, intra mammary immunization, T cells

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Introduction: The prevention of breast cancer is an active area of research in many countries and several different agents have been examined, notably estrogen antagonists. While these agents can be effective in high risk individuals, they only reduce the risk of specific subtypes of cancer and moreover these currently available drugs are associated with significant side effects and must be consumed daily and over years. Thus, an alternative approach to primary prevention of breast cancer is to train the mammary gland mucosal immune system to recognize and destroy potentially malignant cells based on overexpression of known tumor antigens. Rationale for this approach comes from the fact that breast cancer originates from a mucosal tissue which is endowed with large number of immune effectors and prior reports based on the effect of commensal bacteria on gut mucosa which suggests that it's feasible to educate gut mucosa in generating effective mucosal immunity. Our overall goal is to develop a preventative vaccination strategy to reduce the incidence and mortality from breast cancer based on training the mucosal immune system to detect potentially malignant cells early in the course of the disease. One important first step towards attaining this unique objective is to have a comprehensive knowledge about the breast mucosal immune system i.e. a profile of immune effectors, and their biology in the breast mucosa, including both normal and hyperplastic breast mucosal tissue. Our goal in the present study is to understand the immune effector biology of breast mucosa (normal and cancer prone) and identify strategies which can enhance the infiltration of antigen-specific immune effectors targeting tumor cells in breast mucosa. As a part of this plan, we intend to use the transgenic mouse model of human breast cancer. We hypothesize that the mucosal immune system of the mammary gland can be modified to detect and eliminate potentially malignant cancer precursor cells. A thorough understanding of the immunity in breast mucosa will enable the design of appropriate vaccination strategies aimed at generating persistent mammary gland homing anti-cancer antigen-specific immune effectors. To test this general hypothesis we propose the following specific aims: **Specific Aim 1:** To determine the immune cell subsets in normal and hyperplastic breast in murine breast cancer models. **Specific Aim 2:** To determine whether immunization of mice with tumor antigen modifies the immune cell subsets and leads to persistent trafficking of tumor antigen-specific T and B cells into the mammary gland. **Specific Aim 3:** Determine an optimal oral vaccine approach able to minimize hyperplasia.

Progress report

Task 1: To determine the immune cell subsets in normal breast mucosa and hyperplastic breast in murine breast cancer models.

The unique feature of gut mucosa is that its immune microenvironment is influenced by commensal bacteria. This indicates that it is amenable to alteration in immune effectors, which play a critical role in maintaining the gut homeostasis. Furthermore, gut associated T cells are involved in not only preventing colonization, but also regulating homeostasis of the epithelial layer. As a part of the mucosal immune system, the mammary gland may have characteristic features similar to that of gut mucosa.

Subtask 1a: To determine if hyperplasia in the breast mucosa modifies the immune microenvironment in affected and unaffected glandular tissue.

Prior studies showed that immune cell density in normal breast tissue is higher than that in benign (hyperplasia and atypical hyperplasia) breast tissue in breast cancer patients. This suggests that the hyperplasia in the breast mucosa might alter the local immune microenvironment. We hypothesize that the hyperplasia in mammary gland may result in the diminution of T cells and other immune effectors in breast mucosa immunity. To address this question, a transgenic mice model BALB/c neu-T mice [1, 2], which expresses the rat Neu gene (RNEU) in breast tissue, was used in this subtask.

First, the presence of the transgene was monitored by PCR and only positive female mice were selected to use as Neu-T Balb/c transgenic mice in this study (see Fig. 1).

Second, 6-12 weeks old Neu-T Balb/c transgenic mice were selected for this experiment, because atypical hyperplasia of breast tissue begins very early (at 4 weeks of age) and develops into spontaneous breast tumors by 15-20 weeks age [3]. To further identify the phenotype of hyperplasia in mammary gland tissues, immunohistochemical assessments of hyperplasia were performed using staining of Ki67 and E-cadherin in fat pads from Neu-T and wild type Balb/c mice.

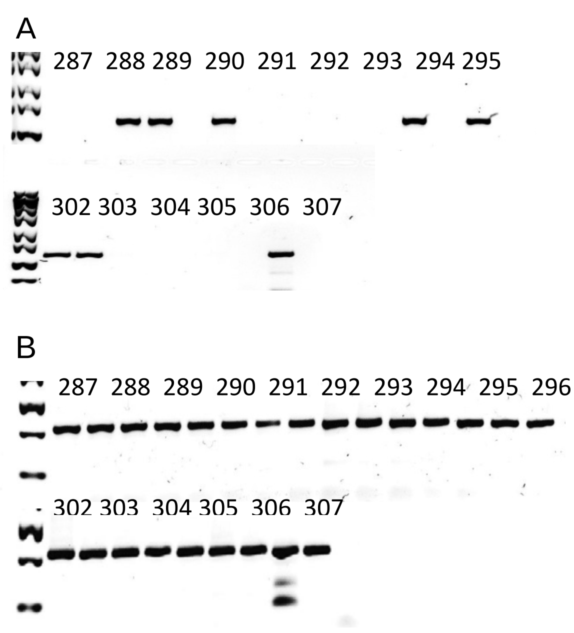


Fig 1. Genotyping mice by PCR amplification was used to identify the Neu-T BALB/c transgenic mice in the murine genome. A. Rat2 Neu-T gene primers were used to check the DNA samples from tails of the mice individually. The positive bands indicate Neu-T transgenic mice. B. Chromosome 3 (IL-2 precursor) primers were used as control to check the DNA samples from the tails of the mice individually.

Last, single cell suspensions obtained from mammary pads and spleens from Neu-T and wild type Balb/c mice were stained with antibodies specific for surface markers of different subsets of immune cells, and analyzed by standard flow cytometry. A minimal mouse sample size of 11 mice per group was used in these experiments and the results are the cumulative data of 3 independent experiments.

Finding #1: The phenotype of hyperplasia in breast mucosa was observed in Neu-T Balb/c transgenic mice.

The proportion of cells stained with the nuclear antigen Ki67 has become the most widely used method for comparing cell proliferation between samples [4]. E-cadherin is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues and loss of E-cadherin has been demonstrated in invasive lobular carcinoma of the breast [5]. In 6-12 weeks old mice, we performed immunohistochemical staining for Ki67 and E-cadherin to identify the phenotype of hyperplasia in transgenic mice. As shown in Fig. 2, the proportion of cells stained with Ki67 in Neu-T mice was more than that in the wild type Balb/c; the lower intensity of E-cadherin staining and the less proportion of E-cadherin-positive cells were detected in Neu-T mice than in the wild type Balb/c. These results demonstrated that the phenotype of hyperplasia in mammary gland was observed in Neu-T transgenic mice but not in wild type mice.

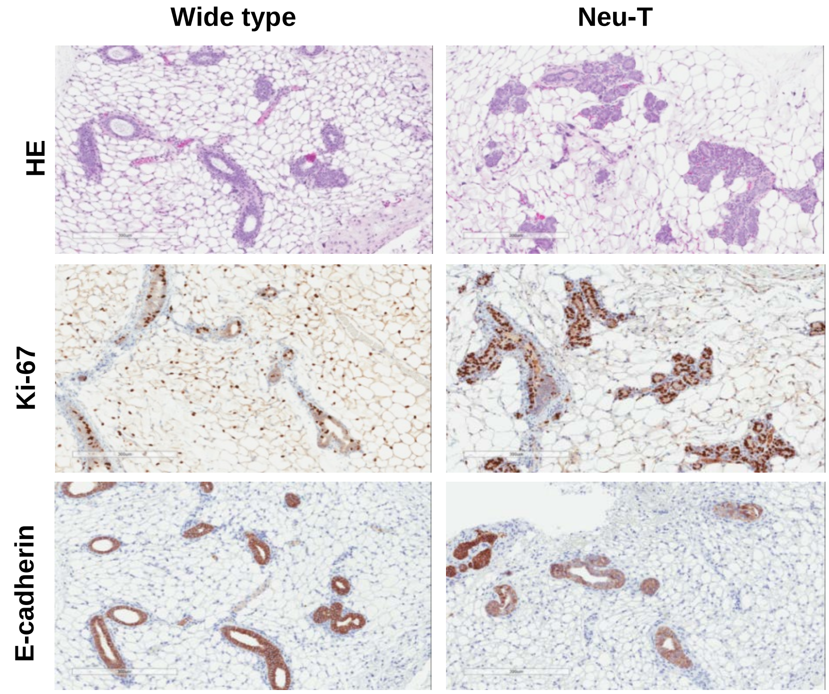


Fig 2. Immunohistochemical assessments of hyperplasia were performed using staining of Ki 67 and E-cadherin in mammary gland tissues. The proportion of cells stained with the nuclear antigen Ki67 in Balb/c Neu-T mice was more than that in the wild type (middle panel); The lower intensity of E-cadherin staining and the less proportion of E-cadherin-positive cells were detected in Balb/c Neu-T mice than in the wild type (lower panel). The pictures shown are the representatives of one of three experiments with similar results. Bar: 300µm.

Finding #2: There are no fluctuations in the mammary gland T cells between wild type and Neu-T Balb/c mice.

Our previous study has demonstrated that the majority of immune cell subset population in the breast mucosa is T cells. Further analysis of T cells subsets revealed that CD4 T cells constituted the bulk of the resident αβTCR cells, and very few standard CD8 T cells were observed in the mammary gland. However, the predominant CD8 T cell population in mammary gland was unconventional CD 8αα + cells (mainly expressed by LELs), which play a critical role in immune surveillance [6]. In the present study, we wanted to determine whether there were any alterations in mammary glands immune T cell subsets between hyperplasia and normal mammary glands. Unexpectedly, our data indicated that the level of total T cells (αβTCR cells) was not significantly altered between wild type and Neu-T Balb/c mice (Fig. 3). Furthermore, the proportion of CD8αα lELs, CD8αβ or CD4 T cells + in mammary glands was not significantly different between wild type and transgenic mice (Fig. 4). This finding is in contrary

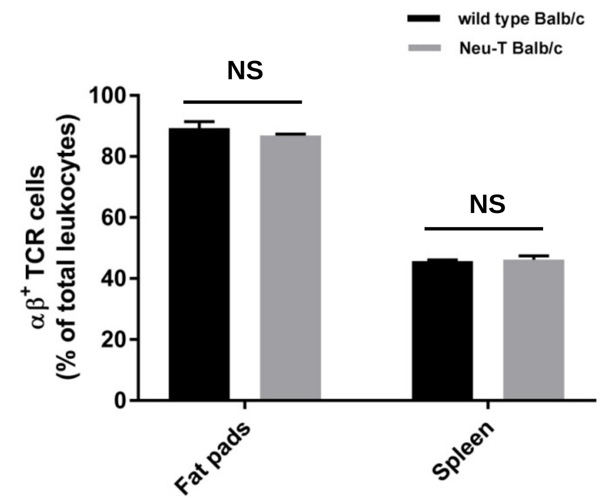


Fig 3. No significant differences are detected about the proportions of αβTCR in the fat pads between the wild type and Neu-T BALB/c mice. Spleen single cell suspensions were stained as control. Single cell suspensions obtained from fat pads and spleens were stained with anti-αβTCR-FITC, and anti-CD45-BV421. The results are shown as mean (±s.e.m) of triplicates and the data shown is the representative of one of three experiments with similar results. NS=No significance.

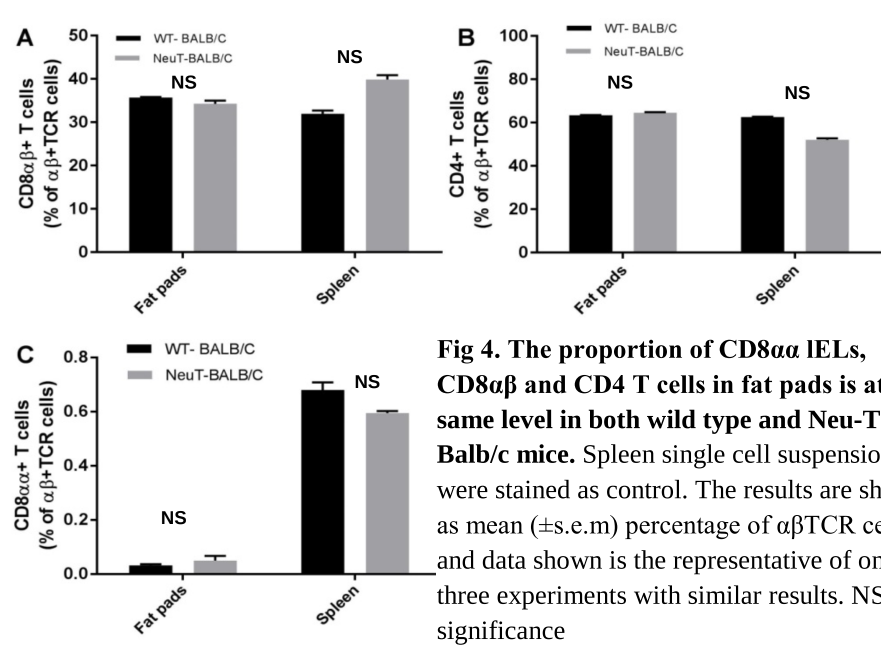


Fig 4. The proportion of CD8αα lELs, CD8αβ and CD4 T cells in fat pads is at the same level in both wild type and Neu-T Balb/c mice. Spleen single cell suspensions were stained as control. The results are shown as mean (±s.e.m) percentage of αβTCR cells, and data shown is the representative of one of three experiments with similar results. NS=No significance

to our hypothesis that the hyperplasia in mammary gland may result in the diminution of T cells and other immune effectors in breast mucosa immunity.

Finding #3: The proportions of myeloid cells in fat pads and spleens are not significantly changed between wild type and Neu-T Balb/c mice.

We further investigated the infiltration of myeloid cells, specifically CD11c dendritic cells (DCs), macrophages, and myeloid-derived suppressor cells (MDSCs) into the mammary glands in wild type and Neu-T Balb/c mice. As shown in Fig. 5, there were no significant differences about the proportions of myeloid cells in fat pads and spleens between wild type and transgenic mice.

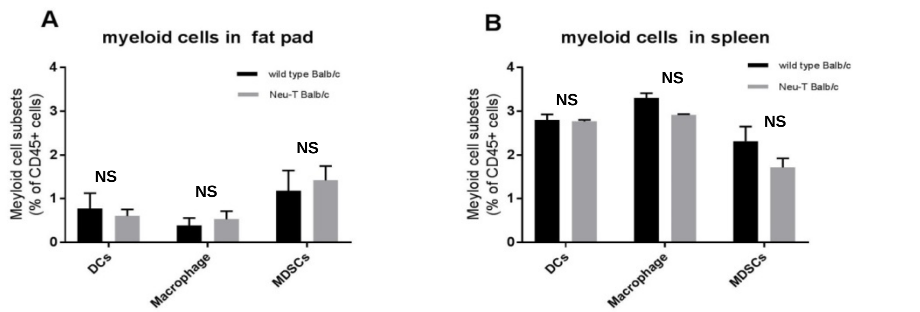


Fig 5. Myeloid cells subsets in wild type Balb/c and Neu-T Balb/c mice. Single cell suspensions obtained from fat pads and spleens were stained with anti-CD11c-APC, anti-F4/80-PECy7, anti-GR1-FITC, anti-CD11b-PE, and anti-CD45-BV421. The expression phenotype of DCs, macrophages and MDSCs in each mouse was determined after gating on CD45+ cells. The results are shown as mean (±s.e.m) percentage of CD45+ cell subsets. Data shown is the representative of one of three experiments with similar results. NS=No significance

Subtask 1b: To determine the effect of age on the immune cell subsets of breast mucosa.

Immunization to achieve optimal immune trafficking is likely to be impacted by those factors such as estrus, pregnancy, lactation, age, etc., which are known to alter the mammary gland biology [7]. In this task, the effect of different conditions (that can effect gland biology) in altering the immune cell subsets of breast mucosa is being determined. In the previous progress report, we already showed that pregnancy and lactation significantly altered immune T cells in mammary glands, whereas there were no fluctuations in mammary gland T cells during the different stages of estrus. Among all the general risk factors of breast cancer, it was reported the strongest risk factor is age [8]. As it is said, the older you are, the higher your absolute risk of breast cancer. In this subtask, our goal is to determine whether age has influence on the mammary gland immune cells subsets. Thus, different ages (7 weeks, 6 months and 10 months old) Balb/c mice were studied, and single cell suspensions obtained from mammary pads and spleens were stained with antibodies specific for surface markers of different immune cell subsets, followed by flow cytometry. A minimal mouse sample size of 11 mice per group was used in these experiments and the results are the cumulative data of 3 independent experiments.

Finding #1: The proportion of CD8αα IELs and CD8αβ T cells decreased significantly in fat pads, whereas the proportion of CD4 T cells remained unchanged in different ages of wild type Balb/c mice.

In this experiment, we evaluated the distribution of T cells in the mammary glands in different ages of mice. As shown in Fig. 6, the relative level of αβTCR cells was not changed in different ages of mice. However, the proportion of CD8αα lELs and CD8αβ T cells in mammary glands decreased significantly, whereas the proportion of CD4 T cells remained unchanged in different ages of wild type Balb/c mice (Fig. 7). As expected, the proportion of the CD8αα lELs and CD8αβ T cells, which act as cytotoxic T cells, decreased in an age-dependent manner.

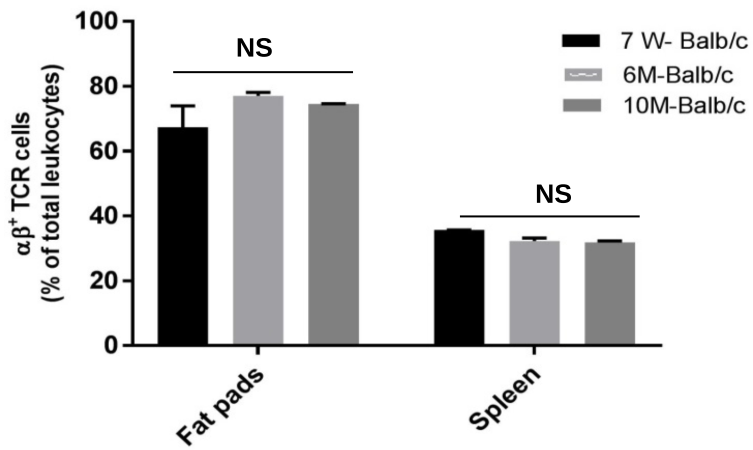
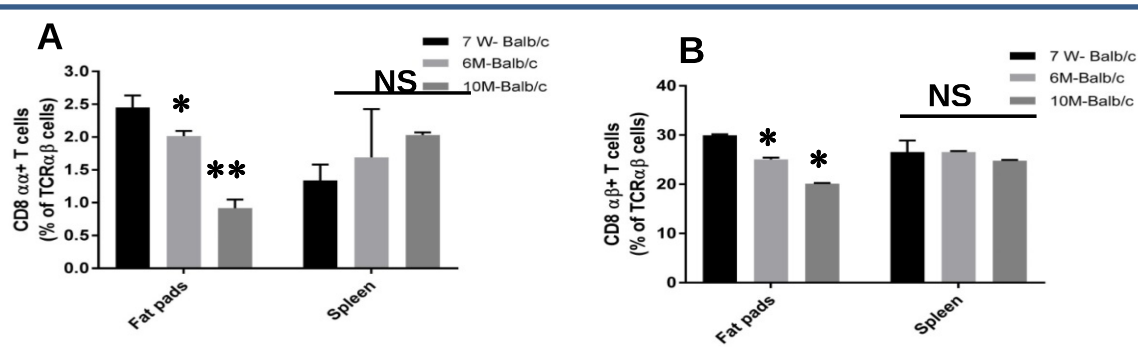


Fig 6. αβ+T cell subsets in different ages of wild type Balb/c mice. Single cell suspensions obtained from fat pads and spleens were stained with anti- αβTCR-FITC and anti-CD45-BV421. The results are shown as mean (±s.e.m) of triplicates , and the data shown is the representative of one of three experiments with similar results. NS=No significance



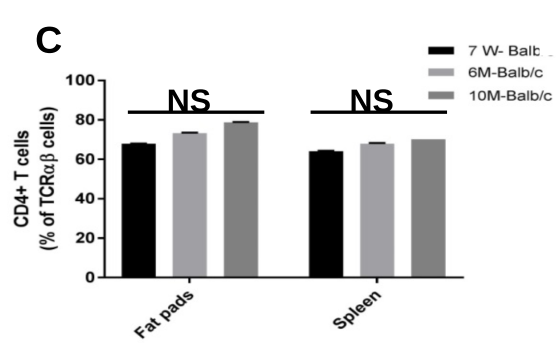


Fig 7. The proportion of CD8αα lELs and CD8αβ T cells decreased significantly in fat pads, whereas the proportion of CD4 T cells remained unchanged in different ages of wild type Balb/c mice. Spleen single cell suspensions were stained as control. The results are shown as mean (±s.e.m) percentage of αβTCR cells and data shown is the representative of one of three experiments with similar results. **∗**indicates $p \leq 0.05$. **∗∗** indicates $p \leq 0.01$.NS=No significance.

Finding #2: The proportions of myeloid cells in fat pads and spleens are not significantly changed in different ages mice.

We continued to investigate the infiltration of myeloid cells, specifically CD11c dendritic cells (DCs), macrophages, and myeloid-derived suppressor cells (MDSCs) into the mammary glands in different ages of mice. As shown in Fig. 8, there were no significant differences about the proportions of myeloid cells in fat pads and spleens between different ages of mice. The results showed that age has no any effect on myeloid cells in the mammary gland.

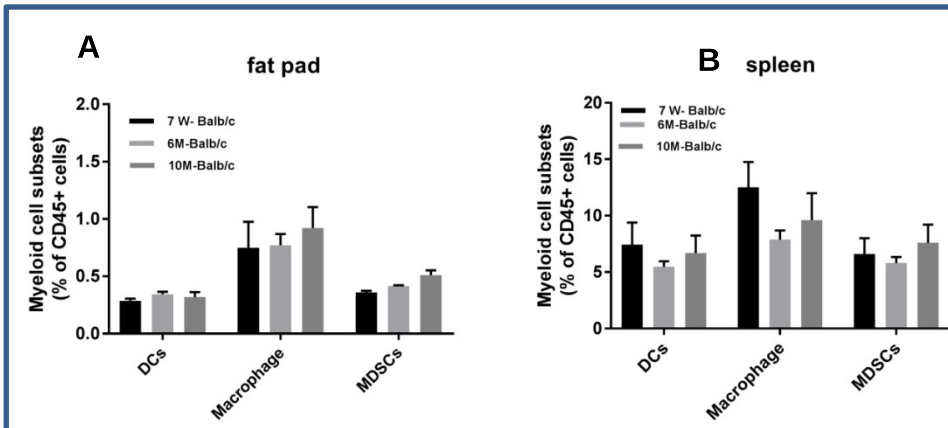


Fig 8. Myeloid cells subsets in different ages of wild type Balb/c mice. Single cell suspensions obtained from fat pads and spleens were stained with anti-CD11c-APC, anti-F4/80-PECy7, anti-GR1-FITC, anti-CD11b-PE, and anti-CD45-BV421. The expression phenotype of DCs, macrophages and MDSCs in each mouse was determined after gating on CD45+ cells. The results are shown as mean (±s.e.m) percentage of CD45+ cell subsets, and data shown is the representative of one of three experiments with similar results.

Task 2: To determine whether immunization leads to sustained lamina propria and intraepithelial immune memory.

In this task, our goal is to determine whether tissue resident memory is established in the glandular tissue by performing a longitudinal analysis following intramammary vaccination. In the previous annual progress report, we showed that intramammary vaccination with peptide antigen leads to sustained immune response lasting up to 8 weeks, whereas no good immune response could be detected when using TMEV encoding the antigens. Currently, we are investigating whether TMEV virus vaccine in combination with peptide vaccine as a prime boost strategy could lead to sustained lamina propria and intraepithelial immune memory. We intramammary immunized wide type Balb/c mice with PBS, empty virus, TMEV vaccine, TMEV vaccine combined peptide vaccine 3 times one week, and observed the immunize response at different time points (1, 4, 8, 16, and 32 weeks).

The Key Research Accomplishments

- Demonstrated that hyperplasia had no any effect of on mammary gland immune cell subsets, which is contrary to our hypothesis.
- The age factor is associated with a decrease in the proportion of CD8 αα lELs as well as CD8αβ T cells in mammary fat pads.

Reportable outcomes:

All new findings in this report are novel and reportable. Manuscripts are in preparation and will be report in major scientific journals.

Presentations at meetings:

None

Publications in scientific journals:

Manuscripts are in preparation and we will submit them to major scientific journals.

Conclusions:

Our goal is to develop preventative breast cancer vaccine. Given the fact that breast cancer originates from mucosal tissue and it is well known that mucosal tissue associated with gut is endowed with intact immune system, we proposed, in this grant, to study the immune effectors associated with breast mucosa. Based on this information our goal was to develop strategies to educate breast mucosa using different vaccination approaches. In order to stimulate immunity specifically in the breast, we chose in this funding period to explore the distribution of T cells in the mammary gland in wild type, hyperplasia transgenic and different ages animal model, which could reveal the mucosal immunity mechanism and influence immune response to vaccine. We also tried to optimize prime boost vaccination strategies into the breast in order to elicit immunity that persists for longer time following vaccination.

Principal Investigator Statement:

Overall, the progress of this project has been excellent to date. Our staff and I are excited and optimistic about the refinements that we have made to the project. The aims that we have chosen are novel, cutting edge, and challenging to us. The work encompassed in the present report was done exclusively at Mayo Clinic Jacksonville. Based on the encouraging results that were obtained so far in this project (both Aim 1 and Aim 2 are done till now) (Aim 1. Identify the immune cell subsets in normal and hyperplastic breast in murine breast cancer models; Aim 2. Develop an attenuated live virus vaccine targeting multiple tumor antigens and determine whether the immunization with tumor antigen modifies the immune cell subsets and leads to persistent trafficking of tumor antigen-specific T and B cells into breast.) We will finalize the study with testing of oral immunization routes to see if this feasibly generates immunity that can traffic to the mammary gland. All the data are expected to result in a publication and enable access to future funding testing the novel idea that we can prevent breast cancer by immunizing into the gut or directly into the mammary gland.

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Appendices :

None